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EVOLUTION OF THE VERTEBRATE PINEAL GLAND: THE AANAT HYPOTHESIS

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The defining feature of the pineal gland is the capacity to function as a melatonin factory that operates on a ~24 h schedule, reflecting the unique synthetic capacities of the pinealocyte. Melatonin synthesis is typically elevated at night and serves to provide the organism with a signal of nighttime. Melatonin levels can be viewed as hands of the clock. Issues relating to the evolutionary events leading up to the immergence of this system have not received significant attention. When did melatonin synthesis appear in the evolutionary line leading to vertebrates? When did a distinct pineal gland first appear? What were the forces driving this evolutionary trend? As more knowledge has grown about the pinealocyte and the relationship it has to retinal photoreceptors, it has become possible to generate a plausible hypothesis to explain how the pineal gland and the melatonin rhythm evolved. At the heart of the hypothesis is the melatonin rhythm enzyme arylalkylamine N-acetyltransferase (AANAT). The advances supporting the hypothesis will be reviewed here and expanded beyond the original foundation; the hypothesis and its implications will be addressed.

Keywords Pineal, Evolution, Melatonin, Retina, AANAT, Vertebrates

VERTEBRATE MELATONIN RHYTHM GENERATING SYSTEMS

It is appropriate to consider the general features of melatonin rhythm generating systems (Klein et al., 1998), before entering into an evolutionary discussion (Figure 1). The term 'melatonin rhythm generating system' was created to emphasize that the rhythmic generation of melatonin in mammals requires other structures (Klein, 1982). Although this is not the case in lower vertebrates in which pinealocytes are self-contained,

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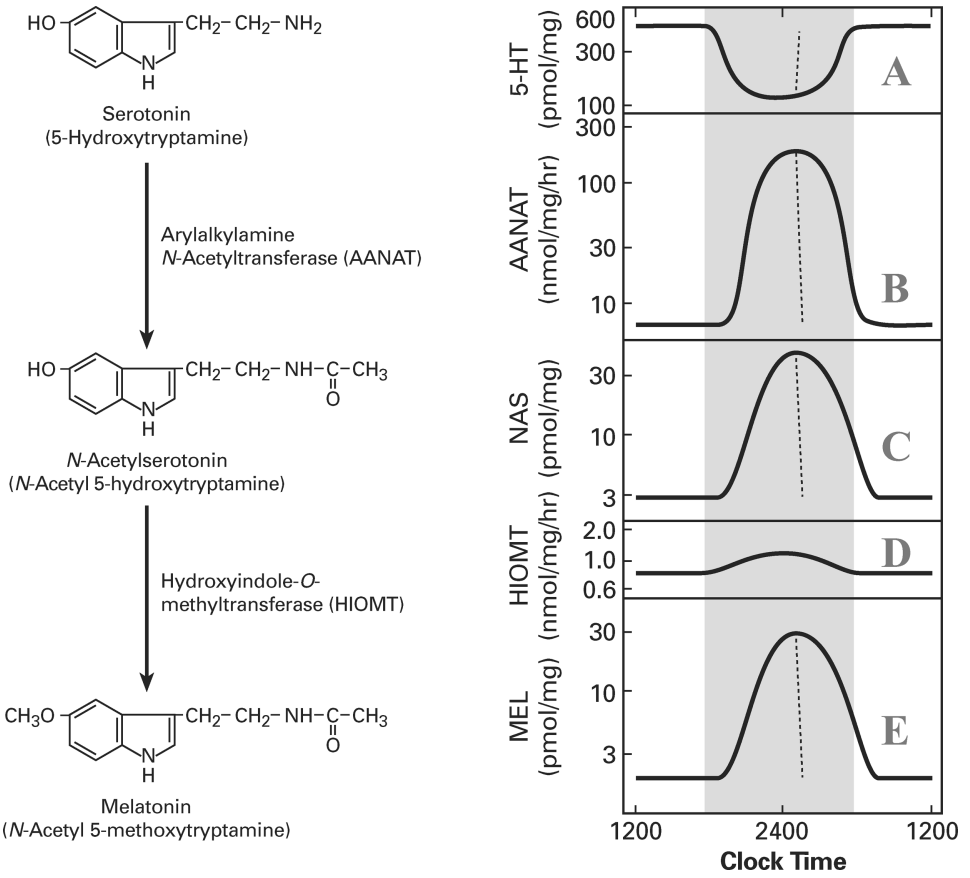

FIGURE 1 Daily changes in the conversion of serotonin to melatonin in the pineal gland. The dotted line represents changes which occur following exposure to light at night.

melatonin rhythm generating systems (Zatz, 1989; Falcon, 1999), use of the term is important because it emphasizes the complex multicomponent nature of these systems and the advances made in understanding their function. It also is a reminder of the unanswered questions regarding how the complex mammalian melatonin rhythm generating system evolved, how it becomes organized during development, and how it functions.

We can disregard the details of these organizational differences, because it is more relevant here to concentrate on the common features of all melatonin rhythm generating systems, because they are likely to reflect the ancestral system. As indicated above, the most obvious is the pattern of melatonin synthesis, *i.e.*, melatonin production is always elevated at night. All organisms can tell time using this signal, but each may use the signal differently. This is apparent because melatonin does

not have a single defining biological effect. For example, well recognized effects of melatonin are seen in some systems but not in others: melatonin promotes sleep in some, but not all, animals; it inhibits reproduction and promotes weight gain in some, but not all, animals; and it stimulates locomotor activity in some, but not all, animals. Accordingly, melatonin is best viewed only as an indicator of time which is used by different species to their own advantage.

Another common feature of melatonin rhythm generating systems is that the melatonin rhythm is driven by a circadian clock, with the exception of salmonoid fishes (Falcon et al., 2003). The circadian clock gives vertebrates the advantage of being independent of the environmental lighting cycle and allows it to predict when events will occur (dawn and dusk) and to tell time (night versus day). Clock-dependent control of downstream functions also prevents inappropriate activation of physiological events simply by exposure to darkness. For example, it prevents burrowing animals that are typically active during the night from being active during the day when in a dark burrow. Circadian clock-control of melatonin production is correctly viewed as unifying vertebrate biological systems, because the light/dark = melatonin rhythm signal is used by most to fine tune daily and annual rhythms.

A third conserved feature of melatonin rhythm generating systems is that light fine tunes circadian clock function. Two modes of action are involved. One is to modify the circadian clock so that it is synchronized with the environmental lighting cycle; as a result, the melatonin rhythm is turned on and off in synchrony with dawn and dusk. This effect of light modifies the circadian clock so that it reflects seasonal changes in day length. A second effect of light is to act downstream of the circadian clock to block clock-stimulation of downstream events (Klein and Weller, 1972; Moore and Klein, 1974; Klein and Moore, 1979). This gating function of light, also referred to as a masking function, tailors circadian stimulation of melatonin production to precisely mirror changes in lighting. As a result, the melatonin rhythm reflects both the circadian clock and environmental lighting.

An additional highly conserved feature of melatonin rhythm generating systems is molecular in nature, specifically, the role that cyclic AMP plays in controlling AANAT activity (Klein et al., 2002, 2003; Ganguly et al., 2001, 2002a, 2002b). Cyclic AMP acts through a protein kinase A system to phosphorylate two highly conserved protein kinase A motifs located in the N- and C-terminal regions which flank the catalytic core (Ganguly et al., 2005). This leads to binding to 14-3-3 proteins, resulting in activation and stabilization of AANAT, which in turn causes increase in melatonin production. Light reverses this, leading to a decrease in AANAT associated with 14-3-3 and subsequent proteolytic destruction of unbound AANAT. Cyclic AMP in pinealocytes from different species

may be controlled by different mechanisms; however, the effects of cyclic AMP on AANAT are highly conserved. The above common features of vertebrate melatonin rhythm generating systems are important to consider in an evolutionary context, because their conservation is proof of their importance.

In contrast to these conserved features, distinct class- and species-specific differences exist, which reflect class-specific innovation, genetic experimentation, and different degrees of success. Differences in pineal glands among vertebrates have been given significant attention (Eksröm and Meissl, 2003; Collin and Oksche, 1981; Klein et al., 1998). In submammals, including fish and birds, the clock, phototransduction mechanisms, and melatonin synthesis are located in the pinealocyte (Cassone, 1991; Falcon et al., 2003; Iuvone et al., 2005). In mammals, however, the clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore and Klein, 1974; Klein and Moore, 1979; Klein, 1985), which appears to reflect the advantage of the centralization of the clock (Klein et al., 1991). The SCN communicates with the pineal gland by a multisynaptic neural pathway that passes through central and peripheral structures. Melatonin production is turned on at night as a result of SCN stimulation of norepinephrine release from sympathetic nerve terminals in the gland. Norepinephrine has a positive effect on melatonin production, mediated by α_1- and β_1-adrenergic stimulation of cyclic AMP. Light acts on the mammalian melatonin rhythm generating system via the retina and a retinohypothalamic projection to entrain the SCN clock; light also acts via this projection downstream of the clock to gate neural stimulation of the pineal gland.

Although the mammalian versus submammalian division of melatonin rhythm-generating systems is convenient, it is not entirely accurate because the bird system appears to be a hybrid: Light acts via two routes. One involves photodetector elements of pinealocytes and the second involves the retina and a neural circuit which includes the avian homolog of the SCN to suppress melatonin production during the day (Cassone, 1991). This action is mediated by the release of norepinephrine, which acts via α_2-adrenergic receptors via an inhibitory mechanism to inhibit melatonin production (Pratt and Takahashi, 1987). In this regard, norepinephrine has opposite effects from those in mammals. The redundant nature of these two systems may reflect the necessity to maintain low levels of melatonin during the day in birds.

Although fascinating, the differences among pinealocytes and melatonin rhythm generating systems will be put aside here so that focus can be placed on the most highly conserved feature of the vertebrate pineal gland–the daily rhythm in melatonin production–and on the question of what factors led to this and the evolution of the pinealocyte.

THE PINEALOCYTE AND RETINAL PHOTORECEPTOR: VARIATIONS ON A THEME

Abundant evidence from several disciplines reveals that many distinctive features are shared by the pinealocyte and retinal photoreceptor. This body of evidence grew rapidly in the last quarter of the 20th century (O'Brien and Klein, 1986).

Functional evidence from ablation studies gave indirect support to suggest the submammalian pineal gland detected light. However, it was not until Eberhard Dodt and his colleagues used electrophysiological methods to demonstrate that fish and anuran pinealocytes detect light was the photosensitive capacity of the pinealocyte established (Dodt, 1963; Morita and Dodt, 1975; Dodt and Meissl, 1982). Subsequently, direct effects of light on isolated avian and fish pineal glands and pinealocytes in culture were described in studies of the melatonin pathway (Rosner et al., 1971; Binkley et al., 1978; Gern and Ralph, 1979; Wainwright and Wainwright, 1979; Hamm et al., 1983; Zatz et al., 1988, 1989; Falcon et al., 1989).

Equally important was the demonstration that the retina shared the defining feature of the pineal gland – the capacity to make melatonin (Cahill et al., 1991; Iuvone et al., 2005). Although melatonin production in the retina is very low, compared to the pineal gland, it is clear that the genes encoding the two enzymes dedicated to melatonin synthesis– AANAT and HIOMT – are expressed in the retinas of many, albeit not all, vertebrates. Melatonin is thought to improve photoadaptation by the retina. In many cases, melatonin is synthesized locally for this purpose; however, in cases where melatonin does not appear to be synthesized, including the monkey, human, and cow (Wiechmann, 1993; Rodriguez et al., 1994; Bernard et al., 1995; Craft et al., 1999; Coon et al., 1996), pineal-derived melatonin could act in place of locally synthesized melatonin through high affinity melatonin receptors (Reppert, 1997; Barrett et al., 2003; Dubocovich et al., 2003).

Anatomical evidence of the similarity of the submammalian pinealocyte and retinal photoreceptor became obvious from structural studies that described the photoreceptor elements of pinealocytes and identified the common features shared with the vertebrate retinal photoreceptor, including a highly folded membranous outer segment, a 9+0 cilium, and a polarized organization, with a distinct cell body and centrosome (Eakin, 1973; Collin and Oksche 1981; Ekström and Meissl, 2003; Klein, 2004).

The anatomical similarity of the pineal and retinal photoreceptor has also become evident from developmental studies of mammalian pinealocytes that transiently exhibit photoreceptor features (Zimmerman and Tso, 1975); the adult pineal gland, however, does not retain these features and is not photosensitive.

Genetic evidence of the similarities of the pineal gland and retinal photoreceptor started to appear early in the 1970's with the use of antisera against proteins found in the retinal photoreceptors (Kalsow and Wacker, 1977, 1978). Measurements of both protein and mRNA subsequently established that many genes required for retinal phototransduction proteins are highly abundant in the mammalian pineal gland, including S-antigen, phosducin (MEKA), interphotoreceptor retinoid binding protein, opsin kinase, and recoverin (Somers and Klein, 1984; Korf et al., 1985, 1992a, 1992b; Mirshahi et al., 1985; Donoso et al., 1985; van Veen et al., 1986a, 1986b, 1986c; Rodrigues et al., 1986; Reig et al., 1990; Schaad et al., 1991; Babila et al., 1992; Blackshaw and Snyder, 1997). In mammals, opsin is not expressed at significant levels, suggesting the highly expressed phototransduction genes function in G-protein mediated transduction in the pinealocyte. It appears that opsin was replaced by other G-protein regulated receptors, most notably adrenergic and VIP receptors. Analysis of photosensitive pinealocytes of fish revealed that pineal-specific forms of opsin exist (Okano et al., 1994; Max et al., 1995; Mano et al., 1999; Kojima and Fukada, 1999) indicating the fish pineal gland evolved to meet different selective pressures than those which directed retinal photoreceptor evolution.

Photoreceptor potency has been reported for anuran, avian, and mammalian pinealoyctes (Araki, 2001; Araki et al., 1988, 1993; Tosini et al., 2000; Shimauchi et al., 2002; Jangir et al., 2005). During development, these cells have the potential to develop enhanced photoreceptor features in culture and *in vivo* in response to selected agents and transplantation regimens, revealing that at an early point in development fate can be shifted from a pinealocyte to retinal photoreceptor phenotype.

Developmental control mechanisms are shared by the pinealocyte and retinal photoreceptor. Impressive molecular evidence is now accumulating which clearly demonstrates that the development of both involves similar transcription factor cascades and that promoters of genes encoding proteins dedicated to phototransduction and melatonin contain a variation of a sequence described as the photoreceptor-conserved element (Chen et al., 1997; Furukawa et al., 1999; Gamse et al., 2000; Bernard et al., 2001; Ekström and Meissl, 2002; Appelbaum et al., 2004, 2005). If the expression of OTX2, a gene required for normal retinal development, is knocked out in the mouse, the pineal gland does not develop (Nishida et al., 2003).

Brain tumors of the type described as trilateral retinoblastoma also attest to the link between the pinealocyte and retinal photoreceptor. The trilateral retinoblastoma is a form of retinoblastoma in which a midline tumor develops in the pineal region of subjects with uni- or bilateral retinoblastomas (Jakobiec et al., 1977; Bader et al., 1970; Mourotova, 2005).

Embryological investigations have also established that both the pineal gland and retina develop from neighboring regions of the neural plate

(Calvo and Boya, 1981; Rubenstein et al., 1998), as reviewed in detail by Ekström and Meissl (2003). *"Variations on a theme,"* seems to be a valid way to describe the pinealocyte and retinal photoreceptor. Although it is clear they have become specialized, with respect to their relative capacity to produce melatonin and detect light, the concept that both evolved from a single photosensitive melatonin synthesizing cell seems obvious (Figure 2).

A COMMON ANCESTRAL PHOTODETECTOR

The precise features and characteristics of this ancestral cell are difficult to establish, because there is no extant example. An approximation can be made by examination of the photoreceptor cell of the subvertebrate chordate, the urochordate *Ciona*. Recent genome analysis has established that *Ciona* is a chordate and that the genome contains many phototransduction genes found in vertebrates.

Some of these phototransduction genes are expressed in or near photodetector cells in the sensory vesicle of the *Ciona* tadpole (Kusakabe et al., 2001; Inada et al., 2003; Horie et al., 2005). Structural analysis revealed this cell is remarkably similar to the vertebrate photodetector (Eakin, 1973). Accordingly, it is highly likely the general architecture of the retinal photoreceptors and the pinealocyte of submammals is found in the photodetector of *Ciona*. Moreover, although *Ciona* is not in the direct line leading to vertebrates, it shares a common ancestor with vertebrates. Therefore, the *Ciona* photoreceptor appears to represent the general structure of the ancestral cell which evolved into the vertebrate pinealocyte and retinal photoreceptor.

Genome analysis of *Ciona* has failed to identify the presence of genes encoding synthesis AANAT, HIOMT, and melatonin receptors (Klein, 2004), suggesting the ancestral vertebrate photodetector did not make melatonin and that the requisite genes were acquired later.

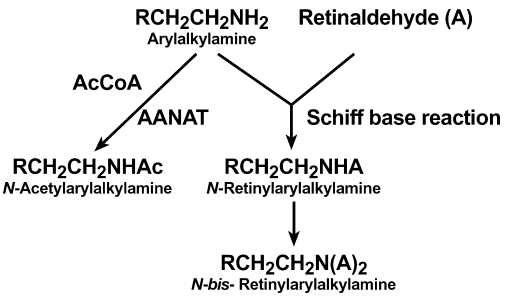

FIGURE 2 Hypothetical inhibitory effect of AANAT on the interaction of arylalkylamines and retinaldehyde(A) in the retina.

THE ACQUISITION OF AANAT

The earliest presence of an ancestral vertebrate AANAT among chordates is in amphioxus (Coon and Klein, 2006). It is not clear whether it was acquired by the normal mechanism – vertical transmission – or if was acquired by lateral transfer (Iyer et al., 2004). In the first case, the absence from the *Ciona* genome might reflect gene loss. In the case of lateral transfer, AANAT might have been acquired from bacteria.

In either case, the appearance of AANAT in the chordate line at the cephalochordate level may represent the first step towards both melatonin synthesis and the evolution of the pineal gland. The synthesis of melatonin in vertebrates differs from that elsewhere in that the specific vertebrate genes are absent from arthropods, nematodes, and higher plants. In the case of acetylation, there is no evidence of rhythmicity associated with vertebrate AANAT. The synthesis of melatonin outside of vertebrates (Hardeland and Poeggeler, 2003) could reflect the action of other enzymes with overlapping substrate selectivity. It should be added that it is unlikely that melatonin receptors played a selective role in the development of the capacity to make melatonin, because melatonin receptors have not been reported outside of vertebrates. This suggests that melatonin synthesis was not the original function of AANAT in the vertebrate photoreceptor.

The Advantage of AANAT in Photoreceptors

Several characteristics of AANAT and of retinal biochemistry can serve as guides in attempting to understand the advantage the acquisition of AANAT served. First, AANAT can broadly acetylate arylalkylamines (Voisin et al., 1984; Coon et al., 1995; Ferry et al., 2000, 2004; Klein and Coon, 2006). Second, AANAT occurs at significant levels only in photodetector-derived cells, the pinealocyte, and retinal photoreceptor (Klein et al., 1997). Third, the substrates of AANAT as amines can react with retinaldehyde–the essential chemical of the visual cycle–via Schiff base formation (Klein, 2004) (Figure 3); the importance of retinaldehyde to photodetection is that one molecule of 11-*cis*-retinaldehyde is converted to the inactive all-trans form when a photon is captured. The reaction of amines with retinaldehyde can theoretically lead to depletion of retinaldehyde and formation of cytotoxic *bis*-retinyl arylalkylamines (Klein, 2004). Moreover, a related class of aromatic amines, the arylamines, are known to be retinotoxic and to act via Schiff base formation, which in turn short circuits the visual cycle and prevents accumulation of 11-*cis*-retinaldehyde (Goodwin et al., 1957; Bernstein and Rando, 1985; Bernstein et al., 1986).

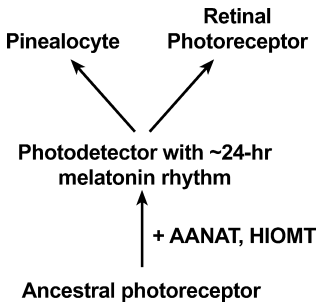

FIGURE 3 Outline of the evolution of the pinealocyte and retinal photoreceptor from a common ancestral photoreceptor.

AANAT would prevent these effects of arylalkylamines because N-acetylation renders the amine inactive, preventing further reactions unless a deacetylating enzyme is present to regenerate the amine. Accordingly, AANAT can be viewed as a detoxification agent, much as arylamine N-acetyltransferase is viewed in the liver and other tissues, where it inactivates arylamines by N-acetylation (Hein et al., 2000; Hein, 2000; Kilbane et al., 1991; Weber, 1971, 1972, 1987). In addition to inactivating the amine, N-acetylation also neutralizes the strong charge on the molecule imparted by the amine, thereby facilitating the elimination of the compound by enhancing solubility in the lipid membranes of the cell.

EVOLUTION OF THE MELATONIN SIGNAL

Melatonin synthesis is likely to have evolved in steps, including the addition of hydroxyindole-*O*-methyltransferase (HIOMT). Like AANAT, it also would serve a detoxification function because O-methylation blocks further modifications of hydroxyl groups leading to more reactive derivatives. In addition, O-methylation increases the lipid solubility of aromatic groups because methoxy derivatives have greater solubility in cell membranes.

In the initial stages of evolution of melatonin synthesis, serotonin may not have been abundant. Accordingly, AANAT and HIOMT may have played a more general role in detoxification, rather than being dedicated to melatonin synthesis. However, the abundance of serotonin would increase as serotonin evolved into a useful transmitter.

Daily rhythmic changes in melatonin production could have evolved from a pressure to enhance photodetection at night: increased detoxification of aromatic amines at night could enhance the available 11-*cis*-retinaldehyde. Enhanced photodetection would provide a competitive advantage to organisms at this point in evolution, when life was evolving in an aqueous environment, which is likely to have been dark and

murky. With the challenge of low light levels, especially at night, any mechanism which increased photosensitivity would be valuable and provide a selective competitive advantage. The increased capacity to detect light in the murky primordial environment would give the ancestral vertebrate greater capacity to detect and evade predators, navigate, and seek and capture food in less populated darker niches.

Melatonin signaling may have resulted from the nonspecific activation of receptors that had evolved to detect other compounds. Melatonin was unusual because it was providing a time signal, which would be of importance in synchronizing the organism with the 24 h photic environment. The daily rhythm in melatonin may have synchronized all physiological functions.

Conflict Between the Two Visual Chemistries

According to the above, the two chemistries which are unique to photodetectors, the retinoid cycle and melatonin synthesis, are in conflict. The driving force of evolution to improve, in the context of the photoreceptor, involved improved capacity to both synthesize melatonin and to detect light. Higher levels of melatonin production required higher amounts of serotonin. This was at odds with the pressure to increase photodetection by increasing the abundance of 11-*cis*-retinaldehyde because of the amine-aldehyde reaction and formation of Schiff bases depleted retinaldehyde.

Resolution of the Conflict

The solution to the melatonin synthesis/retinoid conflict was to segregate each into separate cells, one being the antecedent of the pinealocyte and the other the antecedent of the retinal photoreceptor. With this divergent evolutionary trend, it was possible for photodetection to continue to improve without the conflict imposed by high levels of serotonin, and it was possible for melatonin synthesis to continue to evolve in organisms which had a better capacity to detect light–in retinal photoreceptor antecedents.

AANAT IN THE RETINA

Melatonin synthesis is generally thought to be the function of AANAT in the retina. This is certainly the case in many retinas. However, several observations suggest that AANAT serves a second function in the retina. There are at least two forms in fish, one preferentially expressed in the retina and another in the pineal gland (Coon et al., 1999). This kind of genetic evolution has not occurred in other classes. However, the lesson from fish is that AANAT may serve a function in the retina other than

melatonin synthesis. The function in the retina may involve the primitive function of AANAT–detoxification. This may explain why the retinal form of fish AANAT retains a broader specificity; whereas, that in the pineal is more narrowly specific for serotonin. Another curious situation regarding retinal AANAT occurs in primates and ungulates, where AANAT is very highly expressed in the retina; whereas, HIOMT is not detectable (Coon et al., 2002). In this case, it is possible that melatonin in the retina is devoted primarily to detoxification.

Testing the Hypothesis

Detoxification of arylalkylamines by AANAT within a cellular context will be testable by several approaches, including determining in a cellular system whether AANAT protects against arylalkylamine-dependent deterioration of the retinoid cycle. In addition, it should be possible to determine whether arylalkylamines act in the intact animal to impact the retinoid cycle. It may also be possible to transfect *Ciona* with AANAT to determine if this improves visual detection, especially under arylalkylamine stress.

CLINICAL IMPLICATIONS

AANAT may play a physiological role in amine detoxification, preventing arylalkylamine retinotoxicity, similar to that which occurs with arylamines (Klein and Coon, 2006). Arylalkylamines may also lead to the accumulation of *bis*-retinyl arylalkylamines, which in turn will cause cytotoxic damage leading to macular degeneration, as is seen with *bis*-retinyl ethanolamines (Sparrow et al., 2000, 2003; Ben-Shabbat et al., 2002). Furthermore, genetically inherited and spontaneous mutations in AANAT may increase the susceptibility to arylalkylamine retinotoxicity.

This points to the potential value of developing strategies to enhance the activity of AANAT in the retina. One reasonable approach is to promote 14-3-3 binding of AANAT, thereby preventing degradation of the enzyme and thereby enhancing detoxification of arylalkylamines. It may also be possible to apply lessons learned from the control of expression of *Aanat* to develop strategies to elevate *Aanat* expression and to develop targeted-gene therapies in which highly active forms of AANAT are selectively expressed in retinal photoreceptor cells.

CONCLUSIONS

The AANAT hypothesis of pinealocyte evolution fits with many observations, and is provocative. Many aspects of the hypothesis are highly speculative. However, the hypothesis deserves serious consideration

because of the potential impact it might have on vision, leading to a better understanding of the role AANAT plays in retinal physiology and the development of strategies to enhance the AANAT-dependent detoxification, thereby promoting retinal health.

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